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Vancomycin-induced deletion of the methicillin resistance gene *mecA* in *Staphylococcus aureus*

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***Objective*: To elucidate factors that contribute to the development of vancomycin resistance in methicillin-resistant *Staphylococcus aureus* (MRSA).**

***Methods*: Forty-nine MRSA isolates were subjected to passage selection with vancomycin to isolate mutants with reduced susceptibility to vancomycin. One mutant was chosen for detailed molecular and biochemical characterization.**

***Results*: Five vancomycin-resistant mutants (vancomycin MICs, 6–12 mg/L) were obtained *in vitro* from five MRSA parent isolates. Upon acquisition of vancomycin resistance, all mutants showed a concomitant decrease in oxacillin resistance. In one particular MRSA strain, selection for vancomycin resistance repeatedly produced deletions and rearrangements, including loss of the *mecA* gene. Pleiotropic phenotypical changes, such as yellow pigment formation, loss of haemolysis, thickened cell wall, increased resistance to lysostaphin and reduced cell wall turnover were observed in this mutant.**

***Conclusion*: Acquisition of vancomycin resistance in one MRSA strain triggered *mecA* deletion suggesting that this deletion, coupled to other rearrangements and/or mutations, may be responsible for the increased vancomycin resistance phenotype.**

Keywords: MRSA, staphylococci, vancomycin resistance, *S. aureus*

Introduction

In *Staphylococcus aureus*, resistance to methicillin and related β-lactam antibiotics is encoded by the *mecA* gene, which is carried on a mobile genetic element termed the staphylococcal chromosome cassette *mec* (SCC*mec*). The transfer of this element is mediated by two site-specific recombinases, CcrA and CcrB, which catalyse precise excision of SCC*mec* and its orientation-specific integration into the chromosome of recipient cells.

The stability of SCC*mec* in *S. aureus* is influenced by environmental factors. For example, spontaneous *mecA* deletion has been observed during long-term storage in drug-free medium,[1] and in cultures that have been aged/starved, grown at high temperatures, or given small doses of UV radiation.[2,3] Spontaneous loss of SCC*mec* has also been reported to occur in some lineages of *S. aureus in vivo*.[4,5]

Several reports have implicated a role for *mecA* in the development of MRSA isolates with decreased susceptibility to vancomycin. These studies have demonstrated a correlation between decreased oxacillin resistance with a concomitant increase in resistance to vancomycin either through the deletion of *mecA*[6] or *mecA* mutation.[7]

This study examined 49 clinical MRSA isolates for mutants with decreased susceptibility to vancomycin after passage selection in vancomycin-containing medium. We report here that vancomycin stress repeatedly triggered deletion of *mecA* in one MRSA strain. This event coincided with a decrease in susceptibility to vancomycin and pleiotropic phenotypic changes.

Materials and methods

Strains and culture conditions

The 49 MRSA isolates used in this study were a diverse collection of clinical isolates from the culture collections of this laboratory and the Environmental Science and Research, Communicable Disease Centre, Porirua, New Zealand. *S. aureus* were defined as MRSA with oxacillin MICs ≥4 mg/L, and all were vancomycin-susceptible

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(MIC ≤2 mg/L). All MICs of vancomycin and oxacillin were determined using Etests (AB Biodisk, Solna, Sweden) on Mueller–Hinton agar (MHA-Difco, Detroit, MI, USA) containing 2% NaCl. Disc diffusion sensitivity tests were carried out as described by the National Committee for Clinical Laboratory Standards (NCCLS). Haemolytic activity was sought by spotting 10 μL of a 0.5 McFarland Standard suspension on sheep blood agar plates with incubation for 24 h at 37°C. The resulting growth was observed for their surrounding lytic zones. Presence of β-lactamase was shown with nitrocefin discs (Becton Dickinson, MD, USA).

Selection of mutants with decreased susceptibility to vancomycin

For each of the 49 vancomycin-susceptible MRSA isolates, a single colony was inoculated into 10 mL of tryptic soy broth (TSB) containing 6 mg/L vancomycin and incubated at 37°C until the optical density (OD_{600}, 1 cm light path) was >1.0. The resulting suspension was then diluted to achieve a cell concentration of approximately 100 colonies per 100 μL. Diluted cell suspensions (100 μL) were spread on to tryptic soy agar (TSA) plates containing the same concentration of vancomycin as the broth culture. Plates were then incubated at 37°C until colonies became evident, which took 1–7 days for positive isolates. Colonies were picked from plates that exhibited good growth, excluding pinpoint colonies, and the cycle of broth and agar growth was repeated three times with medium containing the same concentration of vancomycin. With each repeat passage, the time taken for colonies to appear on plates was reduced to approximately 1–2 days for all isolates.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) of *Sma* I-digested DNA was carried out to confirm that clonal identity was maintained between each parent and the respective passaged mutant. PFGE was carried out by contour-clamped homogeneous electric field (CHEF) electrophoresis using the CHEF-DRIII system (Bio-Rad Laboratories, Richmond, CA, USA) as previously described.[8] Gels were routinely run at 6 V/cm, 14°C, at an included angle of 120°, on a 1.2% agarose gel (USB Corporation, Cleveland, OH, USA) with pulse times of 5–35 s for 22 h. The Low Range PFG Marker (New England Biolabs, Inc., Beverly, MA, USA), containing lambda concatemers and *Hin*dIII-digested lambda fragments, was used as a size standard.

All strains were tested for the presence of *mecA* by PCR and Southern blot hybridization using standard protocols and primers as previously described.[8] Primer pair *agr* S3 (5′-GATTTAAGTCGCAGTATTGGT-3′) and *agr* S4 (5′-ACGCGTCATATTTAATTTTGT-3′) were used to amplify a 1.2 kb amplicon containing *agrC* and *agrA*.

Cell wall turnover, autolysis assays, lysostaphin susceptibilities and transmission electron microscopy

Cell wall turnover was determined as described by Hanaki *et al.*[9] using tritium-labelled *N*-acetylglucosamine ([^{3}H]GlcNAc) and measuring the release of radioactive cell wall components. Autolysis activity of whole cells was determined by Triton X-100 treatment as described by Mani *et al.*[10] The cells were incubated at 30°C with shaking and the OD_{620} was measured at 60 min intervals for 4 h. Lysostaphin (1 mg/L)-mediated cell lysis was carried out as described by Peschel *et al.*[11] The cells were incubated at 30°C with shaking and the OD_{620} was measured at 10 min intervals for 30 min. The results shown for all experiments are the mean values of two to four independent experiments that were carried out in triplicate. The standard error of the mean did not differ by more than 15%. For electron microscopy, strains were grown in 10 mL of TSB to the late-log phase of growth to an OD_{600} around 1.0. Cells were pelleted by centrifugation and fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0), with 3 mg/mL Ruthenium Red for 2 h at room temperature. The cells were then washed in 0.1 M sodium cacodylate and mixed for 5 min. This cycle was repeated a further two times. Cells were then post-fixed in 2% osmium tetroxide with 3 mg/mL Ruthenium Red in 0.1 M sodium cacodylate buffer (pH 7.0) for 2 h at room temperature. Three more wash steps were then carried out, and the cells stored at 4°C overnight. Samples were then dehydrated through an ethanol series and propylene-oxide, and embedded in Agar 100 resin (Agar Scientific, Stansted, Essex, UK). The embedded samples were sectioned (80 nm thick) and stained with uranyl acetate and lead citrate. Grids were viewed with a transmission electron microscope Akashi EM-002A at 100 kV accelerating voltage. Mean cell wall measurements were calculated from an average of 30 cells.

Results

Acquisition of reduced susceptibility to vancomycin is concomitant with deletion of mecA in MRSA strain 1126

To elucidate the factors that contribute to the development of vancomycin resistance in MRSA, we subjected 49 clinical MRSA isolates (all vancomycin-susceptible) to passage selection with vancomycin. These 49 isolates consisted of: 15 isolates (oxacillin MICs, ≥128 mg/L) with identical *Sma* I macrorestriction DNA profiles (group I); 24 isolates (oxacillin MICs, 4 to ≥128 mg/L) with PFGE patterns that differed by only one DNA band (group II); and 10 isolates with distinct (≥6 band differences) PFGE patterns (oxacillin MICs, 8 to ≥128 mg/L) (group III). Based on these results, the 49 MRSA isolates selected for this study represented a relatively diverse genetic group.

Upon passage in liquid medium containing 6 mg/L vancomycin, five of 49 MRSA isolates yielded colonies after 1–7 days of incubation (Table 1). Two of the vancomycin-resistant mutants, Vr6-1126a and Vr8-1128a, were derived from group I MRSA isolates, and three (Vr6-1130a, Vr6-1132a and Vr12-1134a) from group II MRSA isolates. The vancomycin MICs for these mutants ranged from 6 to 12 mg/L (Table 1). These mutants were termed with the prefix Vr followed by the number indicating their vancomycin MIC. All corresponding parent isolates were *mecA*-positive, with oxacillin MIC values ≥128 mg/L, and produced β-lactamase. The five Vr mutants exhibited decreased resistance to oxacillin upon acquisition of vancomycin resistance, but remained β-lactamase-positive.

Four of the five vancomycin-resistant mutants were positive for *mecA*, but one mutant, designated Vr6-1126a, had lost *mecA* (Table 1). In three independent experiments its parent MRSA, isolate 1126, yielded mutants that had completely lost oxacillin resistance. In this particular isolate, loss of oxacillin resistance correlated with vancomycin MICs above 4 mg/L. All mutants selected from strain 1126 were non-haemolytic and exhibited a pale yellow pigmentation in contrast with the parent, which was haemolytic and produced white colonies. Their vancomycin resistance remained stable after 10 passages in non-selective medium.

PFGE analysis of mutants derived from strain 1126, and probing with *mecA*, revealed that those with vancomycin MICs ≤4 mg/L had the same PFGE pattern, termed 1a, as the parent

Table 1. Vancomycin and oxacillin MICs for parent *S. aureus* isolates and vancomycin-resistant mutants

		Vancomycin-resistant mutants			
			MIC (mg/L)		
Parent isolate[a]	Oxacillin MIC (mg/L)	Strain	Vancomycin	Oxacillin	*mecA*
1126	≥128	Vr4-1126a	4	48	+
		Vr4-1126b	4	1.5	+
		Vr6-1126a	6	0.05	–
		Vr8-1126a	8	0.2	–
		Vr8-1126b	8	0.2	–
		Vr8-1126c	8	0.1	–
		Vr10-1126a	10	0.1	–
1128	≥128	Vr8-1128a	8	32	+
1130	≥128	Vr6-1130a	6	32	+
1132	≥128	Vr6-1132a	6	48	+
1134	≥128	Vr12-1134a	12	16	+

[a]All parent MRSA were vancomycin-susceptible (MIC <2 mg/L) and positive for *mecA*.

strain (Figure 1, lanes 2, 4 and 5). In contrast, mutants with vancomycin MICs >4 mg/L had lost *mecA*. This loss was associated with large deletions in the SCC*mec*-carrying *Sma* I–G band (Figure 1, lanes 3, and 6–9), resulting either in a smaller additional band I′ (Figure 1, lanes 3, 6, 7, and 9), or in a larger band F′, presumably due to a deletion fusing *Sma* I–G to *Sma*I–I in strain Vr10-1126a (Figure 1, lane 8).

Further analysis using the accessory gene regulator (*agr*) locus as a DNA probe revealed additional rearrangements in the *Sma* I–K band, which carried the *agr* operon (Figure 1, compare panel a and c). According to the published sequence of *S. aureus* N315 genome, *agr* maps in a region distant from the SCC*mec* integration site, suggesting that deletion of SCC*mec* may have induced loss of another genomic element, close to *agr*. This *Sma*I–K band became either slightly larger (Figure 1, lane 3), or became fused presumably to *Sma*I–J band to form a larger band co-migrating with *Sma* I–F (Figure 1, lanes 6–9). These mutants could be divided, based on the size of the deletion and their restriction patterns, into three groups: pattern 1b (21 kb deletion), represented by mutant Vr6-1126a (Figure 1, lane 3); pattern 1c (93 kb deletion), represented by three mutants (Vr8-1126a, Vr8-1126b and Vr8-1126c; Figure 1, lanes 6, 7 and 9), which is similar to 1b, but with loss of *Sma* I–K; and pattern 1d (134 kb deletion), represented by Vr10-1126a (Figure 1, lane 8).

To rule out the possibility that the deletion of *mecA* from strain 1126 was due to a spontaneous event in the absence of vancomycin, 30 individual cultures of 1126 were incubated for 10 days or more in TSB without daily passage at 37°C. Approximately 200 colonies per culture were tested for oxacillin susceptibility. Of 2000 colonies tested, only three mutants (0.15%) with increased susceptibility to oxacillin were isolated. Two of three mutants were negative for *mecA*, but their susceptibility to vancomycin remained unchanged. The third mutant was still positive for *mecA* and susceptible to vancomycin. In contrast, the frequency of *mecA* deletion was 100% in vancomycin-induced, 1126-derived mutants with vancomycin MICs >4 mg/L. Hence, vancomycin triggered or selected for loss of *mecA* at a much higher frequency.

A detailed comparative phenotypic analysis was carried out between Vr6-1126a and its partially-isogenic parent 1126.

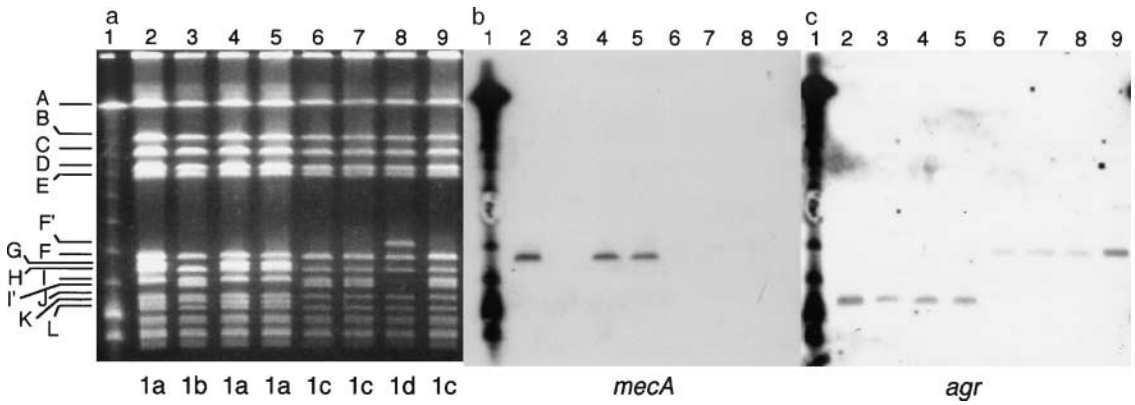

Figure 1. PFGE *Sma* I patterns of vancomycin-resistant mutants derived from parent strain 1126. (a) Lane 1, low range PFG marker (New England Biolabs); lane 2, parent strain 1126; lane 3, Vr6-1126a; lane 4, Vr4-1126a; lane 5, Vr4-1126b; lane 6, Vr8-1126a; lane 7 Vr8-1126b; lane 8, Vr10-1126a; lane 9, Vr8-1126c. The different banding patterns are indicated below each lane. (b) Southern hybridization of panel (a) with *mecA*; and (c) with *agrCA*.

Table 2. Summary of phenotypic properties of MRSA 1126 and Vr6-1126a

Strain/ mutant[a]	Cell wall thickness (nm)	Cell wall turnover (%)[b]	Autolytic activity (%)[c]	Lysostaphin susceptibility (%)[d]
1126 (parent)	26	81	68	5
Vr6-1126a	82	19	74	40

The results shown are the mean values of 2–4 independent experiments. The standard error of the mean did not differ by more than 15%.
[a]1126 and Vr6-1126a were grown in the absence of vancomycin.
[b]Expressed as the percent radioactive cell wall components (CPM) released into the culture supernatant after 90 min resuspension in isotope-free medium containing 100 mg/L of cold *N*-acetylglucosamine.
[c]Expressed as percent remaining OD_{620} at 4 h after resuspension of culture in autolysis assay buffer.
[d]Expressed as percent remaining OD_{620} at 30 min after resuspension of culture in assay buffer with lysostaphin (1 μg/mL). Control tubes incubated without lysostaphin showed less than a 10% decrease in OD_{620} during the time course of the assay.

Mutant Vr6-1126a was more resistant to lysostaphin, and cell wall turnover was markedly reduced in the mutant compared with the parent (Table 2). The cell wall of Vr6-1126a was also three-fold thicker than the parent strain (Table 2), but Triton X-100-induced cell autolysis did not differ significantly.

Discussion

This study shows that vancomycin-induced stress can lead to the deletion of *mecA* in some strains of MRSA. In the strain reported here, this deletion correlated with increased resistance to vancomycin, chromosomal rearrangements and pleiotropic phenotypic changes. Subculturing of MRSA 1126 in non-selective liquid medium gave rise to spontaneous mutants with reduced oxacillin resistance at very low frequency (0.15%). However, subculturing of strain 1126 in vancomycin-containing medium triggered loss of *mecA* at a much higher frequency (100%). Reipert *et al.*[6] identified a spontaneous mutant of methicillin-resistant vancomycin-intermediately-resistant *S. aureus*, where an increase in vancomycin MIC correlated with loss of a β-lactamase-encoding plasmid and a 32.5 kb deletion of SCC*mec*, which extended 65.4 kb into the chromosomal DNA. Hiramatsu *et al.*[1] have hypothesized that penicillinase plasmids may play a role in the stability and phenotypic expression of *mecA*. However, it should be noted that the *mecA* deletion mutants generated in our study remained β-lactamase-positive and therefore appear to be novel.

The size of the *mecA* deletion in 1126 varied depending on the mutant, and rearrangements in other parts of the chromosome (*e.g. agr* locus) were also observed. Some studies have reported that clinical glycopeptide-intermediate *S. aureus* (GISA) isolates are defective in the accessory gene regulator (*agr*) locus.[12] Whilst compromised *agr* function is not an absolute requirement for the development of GISA, it appears to be advantageous for the development of vancomycin hetero-resistance.

The phenotypic properties of Vr6-1126a are consistent with abnormalities in cell wall metabolism, as have been reported for other vancomycin-resistant mutants. The results of this study contribute further to our knowledge on the development of vancomycin resistance in MRSA and we have shown that for one MRSA strain, this results in the deletion of *mecA* at the expense of vancomycin susceptibility. Future work will focus on the apparent incompatibility between oxacillin and vancomycin resistance in this strain.

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